Review

Using *Serratia plymuthica* to control fungal pathogens of plants

David De Vleesschauwer and Monica Höfte*

Address: Laboratory of Phytopathology, Department of Crop Protection, Faculty of Bioscience and Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium.

***Correspondence:** Monica Höfte. Email: monica.hofte@ugent.be

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Abstract

Interest in biological control of plant pathogens has increased in recent years fuelled by trends in agriculture towards greater sustainability and public concerns over the use of hazardous pesticides in the environment. Most studies on the biological control of fungal plant pathogens have tended to focus on the use of antagonistic rhizobacterial strains belonging to the genus *Pseudomonas* or *Bacillus*. However, the development of biocontrol products based on isolates belonging to the Gram-negative genus *Serratia* is now gaining momentum. *Serratia plymuthica* is a ubiquitous bacterium that has been preferentially recovered from rhizospheres all over the world, both as a free-living and endophytic organism. Specific strains of *S. plymuthica* produce a broad palette of antimicrobial compounds and might hold great potential as broad-spectrum biocontrol agents. This review surveys the advances of biocontrol research with respect to plant-associated *S. plymuthica* strains focusing on the principles and mechanisms of action of *S. plymuthica* and their use or potential use for the biological control of fungal plant diseases. A cursory overview of the taxonomy and ecology of *S. plymuthica* is also provided. We highlight recent progress in the identification of antifungal secondary metabolites produced by *S. plymuthica* and pay special attention to the regulatory mechanisms underpinning the production of the latter metabolites. Finally, we discuss several strategies that may provide a basis to improve the efficacy of *S. plymuthica*-mediated biocontrol.

Keywords: *Serratia plymuthica*, Biocontrol, Fungal pathogens, Post-harvest, Mechanisms

Review Methodology: We searched the following databases: CAB Abstracts and Web of Science (keyword search terms used: *Serratia plymuthica*, biocontrol, biological control, post-harvest). In addition, we used the references from the articles obtained by this method to check for additional relevant material.

Taxonomy and Ecology

The genus *Serratia* is named after the Italian physicist Serafino Serrati and belongs to the family Enterobacteriaceae within the Gammaproteobacteria. The only *Serratia* species recognized in the 8th edition of Bergey's Manual was *Serratia marcescens* [1]. In their paper about the taxonomy of *Serratia*, Grimont and co-workers [2] described four species within the genus *Serratia*: *S. marcescens*, *Serratia liquefaciens*, *Serratia plymuthica* and *Serratia marinorubra* (now called *Serratia rubidaea*). Nowadays, recognized species within the genus *Serratia* are *S. marcescens*, the *S. liquefaciens* complex (*S. liquefaciens*, *Serratia proteamaculans* and *Serratia grimesii*) [3]; the so-called 'unusual *Serratia* species [4]: *Serratia ficaria* [5], *Serratia fonticola* [6], *Serratia odorifera* [7], *S. plymuthica*, *S. rubidaea* [8] and *Serratia entomophila* [9]; and *Serratia quinivorans* [10].

The *Serratia* species are ubiquitous and can be found in water, soil, plants and animals (including humans). *Serratia* is an opportunist that has been recognized as a human pathogen only since the 1960s. *S. marcescens* and the *S. liquefaciens* complex are routinely associated with human infections, but also the 'unusual' *Serratia* spp. (except *S. entomophila*) have been described as causing human disease.

S. plymuthica (Lehmann and Neumann [11]) Breed *et al.* [12] has been found in soil [13, 14], water [15, 16] the air of poultry fattening houses [17], insects [15, 18–20] as an opportunistic pathogen in fish [21, 22] and on cold smoked rainbow trout [23] and fresh tuna fish [24]. In addition, *S. plymuthica* has been isolated from blood cultures, surgical wound exudates, the peritoneal fluid, infections of bone marrow, central venous catheters and a human burn site [25–30]. In general, *S. plymuthica* is considered to cause nosocomial infections, which means infections as a result of treatment in the hospital. However, we would like to insert a word of caution on the interpretation of these data. Although *S. plymuthica* has frequently been recovered from the human body, it was rarely isolated as the sole bacterial species present. Based on an international approved German directive (TRBA 466), *S. plymuthica* is nowadays classified into risk group 1 by the DSMZ (German Collection of Micro-organisms and Cell Cultures), indicating that the species does not inadvertently pose a threat to human health. In contrast to *S. marcescens*, which belongs to risk group 2, there is no compelling evidence that *S. plymuthica* is capable of causing human infections. Furthermore, no pathogenicity factors have been identified so far and, in contrast to other nosocomial pathogens like *Burkholderia* and *Stenotrophomonas*, *S. plymuthica* does not inflict disease in alternative animal model systems such as the *Caenorhabditis elegans* assay [31].

S. plymuthica, however, is most frequently associated with plants. This organism has been isolated from the rhizosphere of grass [32], wheat [33], maize [34], oilseed rape [35], grape [36], melon [37], onion [38], *Brassica* sp. [39], *Cichorium intybus* [4], sugarbeet [40], tomato [41] and as an endophyte from the endorhiza of potato [42]. It has been found on the edible parts of green onion, carrot and lettuce [3], on the phyllosphere of spring wheat [43], on *Brassica* spp. [44] and as a contaminant in a raw vegetable processing line [45].

S. plymuthica as a Biocontrol Agent of Fungal Plant Pathogens

Over the last two decades, *S. plymuthica* has received steadily increasing attention as a biological control agent of mainly fungal pathogens. As such, *S. plymuthica* isolates have been used to control fungal soil-borne and leaf pathogens. Furthermore, some papers report the use of *S. plymuthica* to suppress post-harvest diseases. An overview of the *S. plymuthica* strains which have been reported to provide biocontrol of fungal plant pathogens *ad planta* is listed in Table 1.

Using S. plymuthica *to Control Soil-borne Diseases*

Since *S. plymuthica* strains are frequently associated with plant roots, they have most extensively been studied for their ability to control soil-borne fungal diseases.

S. plymuthica strain IC1270 from the rhizosphere of grapes, previously described as *Enterobacter agglomerans* [36] and later on attributed to *S. plymuthica* [46] effectively controlled *Rhizoctonia solani* damping-off of cotton [36], *R. solani* root rot of bean and *Pythium aphanidermatum* pre- and post-emergence damping-off on cucumber [46] under greenhouse conditions. *Pythium* disease severity was reduced to about two-thirds in the IC1270-treated plants compared with control non-bacterized plants.

Over 5000 bacterial isolates from the roots of oilseed rape were screened for antifungal properties against *Verticillium dahliae*. Of the 146 active isolates that were determined, 18 isolates belong to the genus *Serratia* [35]. Of the 18 *Serratia* strains, 16 strains were identified as *S. plymuthica*. All the investigated isolates showed an antifungal activity against *V. dahliae*, *R. solani* and *Sclerotinia sclerotiorum* in bioassays [35]. One of the isolates from this study is the well-characterized *S. plymuthica* strain HRO-C48 (indicated as isolate C48 in [35] and as *S. plymuthica* strain DSMZ12502 in [55, 58]). Dipping strawberry roots in a suspension of *S. plymuthica* HRO-C48 reduced the percentage of *Verticillium* wilt by 18.5% and the percentage of *Phytophtora cactorum* root rot by 33.4%. In three different field trials, *Verticillium* wilt was reduced compared with the non-treated control by an average of 24.2%, whereas the average yield increase was 296%. *Phytophthora* root rot was reduced by an average of 9.6%, while the strawberry yield was increased by 60% compared with the non-treated control [55]. A commercial product on the basis of HRO-C48 has been developed (European patent 98124694.5) and called RhizoStar® (e-nema GmbH, Raisdorf, Germany).

S. plymuthica strain 3Re4-18 (indicated as *S. plymuthica* B4 in [52]) is an endophyte, isolated from the endorhiza of potato [42]. This isolate caused an average reduction of 25% in *R. solani* disease severity in two experiments on potato sprouts. Under field conditions, a disease suppression effect of 31% was achieved on potato, whereas the marketable tuber yield increased by up to 17% compared with the pathogen control. The strain was thus more effective in the field than in the pot experiments. Strain B4 was also used to control bottom rot on lettuce, caused by *R. solani* AG1-1B on leaf discs [53] and in two experimental fields. In both field experiments, soil application with the isolate increased the dry mass of lettuce by as much as 31% and reduced disease severity by 19% [52]. However, strain B4 was not effective in controlling damping-off disease caused by *R. solani* AG4 on sugarbeet seedlings [53].

Alström and Gerhardson [32] describe an isolate of *S. plymuthica* (G15), frequently isolated from roots of various plant species that showed strong antagonism against *Botrytis cinerea* and *Gerlachia nivalis* and moderate antagonism against *R. solani*, *Fusarium culmorum* and

Table 1 Overview of *S. plymuthica* strains providing biocontrol of plant pathogens *ad planta*

Strain	Plant	Pathogen	Reference
IC1270	*Gossypium barbardense* (cotton)	*Rhizoctonia solani*	[36]
	Phaseolus vulgaris (bean)	*R. solani*	[46]
	Cucumis sativus (cucumber)	*Pythium aphanidermatum*	[46]
	Prunus persica (peaches)	*Monilinia fructicola*	[47]
		Rhizopus stolonifer	[47]
	Pirus malus (apples)	*Penicillium expansum*	[47]
	Citrus sinensis (oranges)	*Penicillium digitatum*	[48]
		Penicillium italicum	[48]
	P. vulgaris (bean)	*Colletotrichum lindemuthianum*	[49]
	P. vulgaris (bean)	*Botrytis cinerea*	[49]
	Lycopersicon esculentum (tomato)	*B. cinerea*	[49]
	Oryza sativa (rice)	*Magnaporthe grisea*	(D. De Vleesschauwer and M. Höfte, unpublished results)
IC14	*Citrus sinensis* (oranges)	*Penicillium digitatum*	[48]
		P. italicum	[48]
	C. sativus (cucumber)	*B. cinerea*	[37]
		Sclerotinia sclerotiorum	[37]
CL43	Dutch white cabbage	*B. cinerea*	[44]
		Alternaria brassicicola	[44]
R1GC4	*C. sativus* (cucumber)	*P. aphanidermatum*	[50]
	C. sativus (cucumber)	*Pythium ultimum*	[51]
3Re4-18[1]	*Solanum tuberosum* (potato)	*R. solani*	[52]
	Lactuca sativa (lettuce)	*R. solani*	[53]
	Beta vulgaris (sugar beet)	*R. solani*	[53]
R12	*Fragaria virginiana* (strawberry)	*Verticillium dahliae*	[54]
HRO-C482	*F. virginiana* (strawberry)	*V. dahliae*	[55]
	F. virginiana (strawberry)	*Phytophtora cactorum*	[55]
2-67	*C. sativus* (cucumber)	*Colletotrichum orbiculare*	[56]
	Vitis (grape)	*Eutypa lata*	[57]
B-781	*C. sativus* (cucumber)	*Pythium perplexum*	[13]
A21-4	*Capsicum annuum* (pepper)	*Phytophtora capsici*	[38]

[1]Strain 3Re4-18 was also designated B4.
[2]Strain HRO-C48 has been deposited as DZMZ12502.

Pythium sp. In addition, this isolate significantly increased growth of lettuce plants when applied to the roots under non-sterile conditions.

S. plymuthica strain A153 was isolated from the rhizosphere of wheat [33]. This strain was later on shown to suppress apothecia formation in *S. sclerotiorum* [59]. Inhibition of apothecial formation appears to be due to the production of chlorinated macrolides [59, 60]. Strain A153 has also been used for the biological control of weeds, both in the greenhouse [61] and the field [62].

The isolate *S. plymuthica* R1GC4 (origin could not be retraced) has been tested on rockwool-grown cucumbers for its ability to reduce *Pythium* root rot caused by *P. aphanidermatum*. Strain R1GC4 slightly increased the cumulative cucumber yields [50]. Benhamou *et al.* [51] also used this isolate in a later study in which the defence reactions of cucumber seedlings against *Pythium ultimum* with and without bacterial treatment were studied at the cellular level.

S. plymuthica strain A21-4 was isolated from the roots of onion and significantly inhibited mycelium growth, zoosporangia formation and cystospore germination of *Phytophthora capsici in vitro*. When pepper seedlings were dipped in a cell suspension of A21-4 and transplanted in the greenhouse, the bacteria successfully suppressed *Phytophthora* blight. Disease incidence 60 days after transplanting was 72.4% in the untreated plot, compared with 12.6% in the treated plants. A21-4 readily colonized the pepper roots and the bacterial density on the root was maintained above 10^6 CFU/g root until 3 weeks after transplanting [38].

S. plymuthica strain B-781, which was isolated from a soil sample taken from the Burgundy region of France, effectively controlled damping-off disease of cucumber caused by *Pythium perplexum* [13].

Using S. plymuthica *to Control Fungal Post-harvest Diseases*

Only a few reports deal with the use of *S. plymuthica* to control post-harvest diseases. *S. plymuthica* CL43 (=*S. plymuthica* NCIMB40492), among other bacterial antagonists, has been used to control *B. cinerea* and *Alternaria brassicicola* on Dutch white cabbage at cold store temperature [44, 63, 64]. The *S. plymuthica* strains used

Table 2 Overview of the production of biocontrol-related secondary metabolites by model *S. plymuthica* strains

	S. plymuthica strains					
Metabolite	HRO-C48	IC1270	IC14	A153	R12	3Re4-18
Prodigiosin	–	–	–	ND	ND	ND
Haterumalides	ND	ND	ND	+	ND	ND
Pyrrolnitrin (Prn)	+	+	+	+	ND	ND
Glucanases	–	ND	–	ND	+	+
Chitinases	+	+	+	ND	+	+
Proteases	+	+	+	ND	+	+
Siderophores	+	+	+	ND	ND	+
IAA	+	–	+	ND	ND	–

ND=not determined.

showed *in vitro* and *in vivo* antagonism at 4°C. The use of *S. plymuthica* CL43 and other bacteria to control post-harvest diseases on cabbages is the subject of three different patents (US patents numbers 5780080, 5869038 and 5597565).

S. plymuthica strain IC1270 is an effective antagonist of *Penicillium expansum* (blue mould) on apple, and *Monilia fructicola* on peach [47]. In addition, this strain and *S. plymuthica* strain IC14, isolated from soil around melon roots [37] effectively suppressed *Penicillium digitatum* (green mould) and *Penicillium italicum* (blue mould) on orange [48]. Both strains reduced disease incidence by about 30% compared with control treatments.

Using S. plymuthica *to Control Fungal Leaf Pathogens*

Only *S. plymuthica* strains IC14 and IC1270 have been used for foliar application. Strain IC14 protected cucumber seedlings against *B. cinerea* grey mould and *S. sclerotiorum* white mould diseases of leaves under greenhouse conditions. Disease incidence was reduced by 76 and 84%, respectively [37]. The survival ability of strain IC14 on cucumber leaves is limited, however. The titre of bacteria decreased from 1×10^6 cells per 0.5 cm^2 of leaf tissue to 2.7×10^3 cells per 0.5 cm^2 of leaf tissue after 72 h. Leaf application with strain IC1270 decreased the number of *B. cinerea* spreading lesions from 92% in the control to 64%, and from 78 to 48% in bean and tomato, respectively [49].

Some *S. plymuthica* strains, however, can induce systemic resistance in plants and control leaf pathogens when inoculated on plant roots. Seed treatment with *S. plymuthica* strain 2-67 significantly reduced the number and diameter of lesions caused by *Colletotrichum orbiculare* on cucumber leaves in two of three trials under greenhouse conditions [56]. Soil and seed treatment with *S. plymuthica* strain IC1270 induced systemic resistance to *B. cinerea* on tomato and bean leaves and to *Colletotrichum lindemuthianum* on bean [49]. Effective root colonization resulted in a 35% disease severity reduction. Strain 1270 was also able to induce systemic resistance to *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* on rice, causing reductions in disease severity of as much as 50% (D. De Vleesschauwer and M. Höfte, unpublished results).

Biocontrol Mechanisms

A thorough understanding of the antimicrobial mechanisms employed by *S. plymuthica* is important for an efficient and long-lasting biocontrol. Rhizosphere competence and biocontrol activity of *S. plymuthica* are enabled by antibiosis, parasitism involving production of lytic enzymes, competition for nutrients and iron by secretion of siderophores, and induction of plant defence mechanisms. None of these mechanisms are mutually exclusive and frequently several modes of action are exhibited by a single *S. plymuthica* strain. For instance, the mode of action of strain HRO-C48 comprises a diverse set of biocontrol mechanisms facing both pathogen and host plant [65]. Table 2 presents an overview of the spectrum of biocontrol-associated secondary metabolites produced by model strains of *S. plymuthica*.

Antibiosis

The production of organic antimicrobial secondary metabolites as a biocontrol mechanism of *S. plymuthica* has become increasingly better understood over the past decade. A variety of antibiotics have been identified, including compounds such as pyrrolnitrin (Prn), prodigiosin, the dipeptide antibiotic CB-25-I, 1-acetyl-7-chloro-1-H-indole and haterumalides [32, 35–37, 58, 59, 60, 66, 67].

Few *S. plymuthica* strains produce the non-diffusible red pigment and antifungal antibiotic prodigiosin [32, 35–37, 58]. Pigmented *S. plymuthica* biotypes, which were rarely isolated from plants, seem to be toxic to protozoa [68]. Hence, production of prodigiosin might offer an ecological advantage in widely diverse ecological niches. However, a correlation between the production of prodigiosin and

the level of resistance to several antibiotics, as has been demonstrated for *S. marcescens* [68], could not be confirmed for *S. plymuthica* [58].

The chlorinated macrolides, haterumalide NA, B, NE and X, were among the first polyketide substances found to be produced by isolates belonging to the genus *Serratia* [59, 60]. Isolated haterumalides, purified from the supernatant of *S. plymuthica* strain A153, strongly suppressed apothecial formation, ascospore germination and mycelial growth of several filamentous fungi and oomycetes *in vitro* [60, 69]. Haterumalides NA, B and NE were also isolated from an Okinawan *Ircinia* sponge as inhibitors of the cell division of fertilized sea urchin eggs [70]. Structural similarities to other compounds suggest that the biosynthetic pathway of the haterumalides involves a type I polyketide synthase cluster, similar to the haterumalide biosynthesis in bacteria from the genus *Pseudomonas* [71, 72]. Nevertheless, further research regarding the genetic origin of haterumalides and the underlying biosynthetic pathway is needed to confirm the involvement of a type I polyketide synthase cluster in the biosynthesis of haterumalide antibiotics by *S. plymuthica*.

Prn [3-chloro-4-(2′-nitro-3′-chlorophenyl) pyrrole] is a tryptophan-derived secondary metabolite that has been reported to suppress a wide range of fungal and bacterial pathogens (for review see [73]). Although a vast amount of isolated *S. plymuthica* strains has been demonstrated to produce Prn *in vitro* [35, 37, 60, 67], several studies showed discrepancies regarding the role of Prn in the antagonistic activity of *S. plymuthica* strains. While Prn production was assumed to be a key factor of *S. plymuthica* IC1270-mediated biocontrol of several fungal post-harvest pathogens of peaches and apples [47], no evidence was found for the involvement of Prn in post-harvest control of blue and green mould by the same strain [48]. Likewise, IC1270-triggered resistance against *B. cinerea* was shown to be independent of Prn, whereas Prn was demonstrated to play a prevalent role in direct antagonism towards distinct pathogens by the latter strain [49, 74]. However, these conflicting observations can be reconciled when considering that many biocontrol strains produce a pallet of secondary antimicrobial metabolites and that conditions favouring one compound may not favour another [75]. This varied arsenal of biocontrol traits may enable antagonists to efficiently fine-tune their biocontrol activity and perform their ultimate objective of pathogen suppression under a wide range of environmental conditions. As such, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant diseases by a particular biocontrol agent [76].

Because Prn production is an important biocontrol mechanism against several plant pathogens, extensive work has been carried out to elucidate its gene expression and regulation in the model strain *Pseudomonas fluorescens* Pf-5. In addition to the identification of the Prn biosynthetic gene operon, which comprises four genes [77, 78], Prn has been reported to be under global genetic control by a two-component regulatory system composed of the sensor protein ApdA (also called LemA) [79] and the response regulator GacA [80, 81]. Moreover, a gene necessary for Prn production has been identified as *rpoS*, which encodes the stationary-phase sigma factor sigma(s) [71, 82]. The interactions between the RpoS and the GacS/GacA regulons are, however, poorly understood. Elegant research by Ovadis *et al.* [83] demonstrated the involvement of *rpoS* and *gacA*/*lemA* homologues (tentatively designated *grrA*/*grrS* for global response regulation activator/sensor) in Prn regulation in *S. plymuthica* strain IC1270. Prn-deficient *grrA*, *grrS* and *rpoS* gene replacement mutants were markedly less capable of suppressing *R. solani* and *P. aphanidermatum* under greenhouse conditions, indicating that IC1270-mediated biocontrol is tightly modulated by the GrrA/GrrS global regulatory cascade and the sigma factor RpoS. In addition, Prn biosynthesis was very recently demonstrated to be subject to positive control by a LuxI/LuxR-type quorum-sensing system consisting of an *N*-acyl-homoserine lactone (AHL) synthase (SpII) and an AHL-responsive cognate transcriptional repressor, designated as SpIR [84]. Using an AHL and Prn double-negative mutant of strain HRO-C48, which was deficient in suppressing the growth of several fungal plant pathogens *in vitro*, the authors provided evidence for the involvement of quorum-sensing signalling in biocontrol exerted by *S. plymuthica*.

Parasitism

Parasitism relies on the excretion of extracellular cell wall-degrading enzymes, such as chitinases, proteases and β-1,3-glucanases that can lyse pathogen cell walls [85].

Chitin, an insoluble β-(1,4)-linked polymer of *N*-acetyl D-glucosamine (GlcNAc), is a ubiquitous component of most fungal cell walls. Chitinases, which catalyse the hydrolysis of chitin, can be classified into two major categories. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29) catalyse the progressive release of diacetylchitobiose units starting at the non-reducing end of chitin microfibrils, and *N*-acetyl-β-(1,4)-D-glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc [86]. Based on this system of nomenclature, several types of chitinases have been identified in *S. plymuthica* strains. Strain IC1270 produces two *N*-acetyl-β-D-glucosaminidases of 89 and 67 kDa, an endochitinase with a molecular mass of 59 kDa and a 50 kDa chitobiosidase. Strains IC14

and HRO-C48, on the other hand, have been reported to secrete an endochitinase and a 100 kDa *N*-acetyl-β-1,4-D-hexosaminidase or chitobiase [37, 87].

To date, only one chitinase-encoding gene from *S. plymuthica* has been cloned [88]. Sequencing of the cloned gene *chiA*, which encodes the endochitinase from strain IC1270, yielded an open reading frame coding for 562 amino acids of a 61 kDa precursor protein with a putative leader peptide at the N terminus. Homology modelling of the deduced enzyme's three-dimensional structure revealed high structural similarities with the corresponding enzyme from *S. marcescens*. Both structures consisted of an all-β-strand amino-terminal fibronectin III (FnIII)-type domain, an α+β fold domain, and an α/β-barrel domain. While it has been suggested that the first domain facilitates the binding of chitinase to chitin, the last domain is catalytic, retaining the conserved residues Glu315 and Asp391, which are located in the active site [88]. The antifungal activity of the secreted endochitinase was demonstrated *in vitro* using recombinant DNA techniques. The recombinant strain *Escherichia coli* JM109/pCHITEa1, expressing the *S. plymuthica chiA* gene, acquired the ability to suppress *R. solani* and spore germination of *Fusarium oxysporum* f. sp. *meloni in vitro*. Furthermore, the transformed strain also abrogated root rot disease caused by *R. solani* in cotton seedlings under greenhouse conditions [88].

The 58 kDa endochitinase of *S. plymuthica* IC14 (ChiA) differs from this of strain IC1270 in that it not only hydrolyses chitin but also EGC, a chitin derivative usually used as a test substrate for lysozyme activity, suggesting that IC14 ChiA belongs to the class of bifunctional chitinase/lysozyme enzymes [37]. Such bifunctional enzymes have been suggested to enable bacteria to compete efficiently with fungi and other bacteria in a limited-nutrient environment. Alternatively, broader substrate specificity of chitinases has been related to other aspects of their function such as modulating the intricate relationships between biocontrol bacteria and their host organism [37].

So far, research aimed at elucidating the regulatory mechanisms underlying chitinase production in *S. plymuthica* has been confined to a limited number of strains. The GacS/GacA two-component system has previously been shown to positively regulate the expression of genes coding for secreted enzymes such as chitinases in a group of root-colonizing, plant-beneficial bacteria including *Pseudomonas chlororaphis* PCL1391 and *P. fluorescens* BL915 (for review see [89]). *GrrA* and *grrS* gene replacement mutants of IC1270, however, were deficient in production of the 58 kDa ChiA endochitinase but not in that of the 89 and 67 kDa exochitinases. As the *rpoS* mutant of IC1270 still secretes ChiA, the mutation in *grrA* or *grrS* is unlikely to exert its effect via repression of the stationary sigma factor RpoS, whose expression is positively regulated by the GacS/GacA system in *P. fluorescens* strain Pf-5 [90] and *E. coli* [91]. In addition, regulation of chitinase production seems to act independently of the quorum sensing machinery of IC1270 because synthesis of AHL signal molecules was blocked in both *rpoS* and *grrA*/*grrS* gene replacement mutants [83]. However, Müller *et al.* [65] very recently reported that expression of chitinase is regulated positively by quorum sensing in strain HRO-C48. Likewise, an extracellular chitinase in *S. plymuthica* strain RVH1 is synthesized under the positive control of the SplIR quorum-sensing system [92], suggesting that the regulatory cascades that modulate chitinase production are strain-specific. Recently, it has been demonstrated that the GacS/GacA system partly steers its effects via post-transcriptional control exerted by small regulatory RNAs such as RsmB, RsmZ and RsmY [93, 94]. In *Erwinia carotovora* subsp. *carotovora* and *P. fluorescens*, these regulatory RNAs sequester the RNA-binding protein RsmA and thereby relieve translational repression of target mRNAs. Based on the taxonomic resemblance between *S. plymuthica* and *E. carotovora*, Ovadis *et al.* [83] hypothesize that translation of ChiA mRNAs involves similar post-transcriptional regulators.

Several studies have investigated the role of chitinases in biocontrol activity of *S. plymuthica* strains. Chitinases produced by *S. plymuthica* HRO-C48 played an important role in the antifungal activity of the latter strain both in dual culture assay and *ad planta* [87, 95]. However, chitinolytic activity appears less essential for *S. plymuthica* IC14; when used to suppress *S. sclerotiorum* and *B. cinerea*, synthesis of proteases and other biocontrol traits were involved [37]. Likewise, *S. plymuthica* IC1270-mediated biocontrol against *R. solani* and *P. aphanidermatum* was demonstrated to be independent of chitinase production [74]. Similar results were obtained in IC1270-modulated biocontrol assays with different post-harvest pathogens [47, 48]. Hence, the contribution of chitinolytic activity in *S. plymuthica*-mediated biocontrol is clearly strain-specific and further illustrates the heterogeneous multifaceted character of biocontrol mechanisms employed by distinct bacterial strains against a diverse set of pathogens.

Glucanases and proteases are cell-wall degrading enzymes that are produced by a wide range of *S. plymuthica* strains [35, 37, 42, 53, 96]. However, to date, no studies regarding the regulation or precise role of these antifungal compounds in biocontrol by *S. plymuthica* have been conducted.

Competition for Iron and the Role of Siderophores

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition [97]. Under iron-limiting conditions, bacteria produce a range of low-molecular-weight compounds or siderophores to competitively acquire ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere, thereby depriving pathogenic fungi of this essential element and consequently restricting

their growth [98, 99]. Several *S. plymuthica* strains including IC1270, IC14, 3Re4-18 and HRO-C48, have been shown to secrete potent siderophores *in vitro* when grown on iron-poor media [35, 37, 42, 46, 53, 95]. Additionally, the residual biocontrol activity of distinct Prn- and/or endochitinase-negative mutants of IC1270 has been partially attributed to the unaltered ability of the latter strains to compete for nutrients such as iron [74, 83]. Nevertheless, more detailed studies using siderophore-deficient mutants and application of purified compounds will be required to unequivocally delineate the involvement of bacterial iron chelators in *S. plymuthica*-mediated biocontrol.

Induction of Plant Resistance Mechanisms

An additional mechanism by which *S. plymuthica* can reduce plant diseases is by activating the host plant's defensive repertoire. Although the concept of rhizobacteria-mediated systemic resistance (Induced Systemic Resistance, ISR) has received increasing attention over the last decade, reports about *S. plymuthica* strains mounting systemic resistance are scarce. Gang *et al*. [56] first reported evidence that the *S. plymuthica* strain 2-67 induces ISR in cucumber to *C. orbiculare*. Further evidence showing the ISR-triggering capacity of *S. plymuthica* was provided by Benhamou *et al*. [51]. Using electron microscopy, the authors demonstrated that *Pythium*-challenged induced cucumber root cells undergo significant ultrastructural and biochemical modifications that correlate with the formation of structural barriers that likely prevent pathogen ingress towards the vascular stele accompanied by the deposition of a phenolic-enriched occluding material. Such responses associated with the onset of induced resistance would include the oxidation and polymerization of pre-existing phenols and the synthesis of new phenolic compounds via an activation of the phenylpropanoid pathway. Hence, *S. plymuthica* R1CG4 reduces *Pythium* root rot by priming susceptible cucumbers plants to elaborate a wide range of defence mechanisms. Recently, *S. plymuthica* strain IC1270 was shown to mount ISR against *B. cinerea* and *C. lindemuthianum* in bean and tomato [49]. In rice, however, IC1270 plays an ambivalent role in mounting induced resistance responses. While IC1270 conferred enhanced resistance to *M. grisea* and the bacterial pathogen *X. oryzae* pv. *oryzae*, bacterial colonization significantly promoted subsequent infection with the necrotrophic pathogens *R. solani* and *Bipolaris oryzae*. The differential effectiveness of IC1270 with respect to ISR-mediated disease resistance in rice is most likely due to its capacity to modulate the plant's oxidative machinery. Biochemical and histochemical studies demonstrated that IC1270 primes rice seedlings for a potentiated generation of reactive oxygen species in response to pathogen infection and wounding (D. De Vleesschauwer and M. Höfte, unpublished results).

Phytostimulation

Plant growth is affected by a plethora of abiotic and biotic factors. Most plant growth-promoting rhizobacteria (PGPRs) increase plant growth indirectly either by the suppression of well-established diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens. Alternatively, PGPRs may directly affect plant metabolism resulting in increased plant growth, seed emergence or improved crop yield [85]. Several *S. plymuthica* strains have been demonstrated to exert plant growth-promoting effects in phytochamber, greenhouse and field trials [53, 55, 96]. The plant growth-stimulating ability of such strains has often been linked to their capacity to produce the auxin phytohormone indole-3-acetic acid (IAA) *in vitro*. IAA is the main auxin in plants, controlling many fundamental physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity [100, 101]. However, in several independent studies it was shown that IAA biosynthesis alone cannot account for the overall plant growth-promoting effect of *Azospirillum* [102]. Furthermore, Faltin *et al*. [53] found no correlation between IAA production *in vitro* and the plant growth-promoting effect on lettuce seedlings of several antagonistic bacteria, including the *S. plymuthica* strain 3Re4-18. In view of these data, the growth and yield promotion observed might be explained by the 'additive hypothesis' [103], postulating that growth promotion is the result of multiple coordinated mechanisms such as associative nitrogen fixation, modulation of phytohormonal balances, phytohormone biosynthesis and solubilization of phosphate. Thus, a balanced interplay of different factors including bacterial IAA biosynthesis rather than IAA production *per se* is most likely needed to stimulate plant growth. The use of mutant and transgenic strains and the analysis of inoculants' supernatant might shed more light on the diverse role played by different bacterial factors involved in *S. plymuthica*-mediated phytostimulation.

Conclusions and Future Considerations

Over the past two decades, several *S. plymuthica* strains have been demonstrated to be effective biocontrol agents against soil-borne and foliar diseases. Some *S. plymuthica* strains can also be used to control post-harvest diseases given their ability to antagonize pathogens at cold store temperatures [44, 63, 64]. In addition, *S. plymuthica* strains have been described as entomopathogens [104] and are employed for biological control of weeds [61, 62]. Many strains produce a variety of allelochemicals, including antibiotics, lytic enzymes and iron-chelating siderophores [35, 36, 53, 60, 66, 83]. Moreover, the diverse origin of *S. plymuthica* isolates demonstrates that these bacteria are able to colonize widely diverse ecological niches. Hence, *S. plymuthica* strains might be ideal candidates for use as

broad-spectrum biocontrol agents in integrated crop management.

Despite their potential as low-input practical agents of plant protection, widespread application of *S. plymuthica* strains as commercial biocontrol products has been hampered for several reasons such as the limited number of field tests conducted so far, the difficult formulation of the bacteria, and their emergence as facultative pathogens. Chief among concerns is the often-reported inconsistent performance of biocontrol agents in the field, which is usually attributed to their poor rhizosphere competence [105]. Biocontrol strains can only be used optimally if the molecular basis of their beneficial effects, and the way these traits are influenced by a myriad of biotic and abiotic factors, are unravelled. As many studies have demonstrated discrepancies between the antagonistic potential of the biocontrol agent *in vitro* and its efficacy under field conditions [53], successful reproducible biocontrol on the basis of plant-associated *S. plymuthica* also requires profound knowledge of the ecological and molecular interplay taking place in bacterial communities in order to predict the conditions under which biocontrol can be achieved. Revelations about the modes of action of *S. plymuthica* biocontrol strains will open new doors to design strategies for improving the efficacy of biocontrol products [106]. For instance, identifying different modes of action will facilitate the combination of biocontrol strains to hit pathogens with a broader spectrum of microbial weapons [107, 108]. Identification of key antimicrobials produced by *S. plymuthica*, such as chitinases or Prn, and elucidation of their biosynthetic pathways can be exploited for streamlining biocontrol strain discovery by targeting selection of new isolates that carry relevant biosynthetic genes [109].

Despite the fact that genotypic and phenotypic diversity occurring in natural populations of biocontrol agents provides an enormous resource for improving biological control of plant diseases [110], exploitation of such diversity among bacterial biocontrol agents of fungal plant pathogens has received little attention. Yet, knowledge of the diversity within a group of strains sharing a common biocontrol trait can be exploited to select biocontrol strains that are superior with respect to rhizosphere competence and biocontrol activity. Recent studies by Berg [58] demonstrated that populations of plant-associated and antifungal *S. plymuthica* strains can be highly diverse and thus have great potential for improving biological control. For instance, by matching bacterial genotypes with crops or varieties for which they have a preference, genotypic differences among strains could be exploited to face the biotic and abiotic complexity of natural environments.

A salient feature of *S. plymuthica* is that some strains are able to colonize the endorhiza [42]. Given the intimate relationships with their hosts, endophytic bacteria hold great potential to further our understanding of the multiple facets of disease suppression. As indicated by Compant *et al*. [107], continued work with endophytic bacteria might play a fundamental role in the development of biocontrol agents that are self-perpetuating by colonizing hosts and being transferred to progeny much as is the case with the non-symbiotic endophyte bacterium *Burkholderia phytofirmans* PsJN [111] or associative nitrogen-fixing bacteria on sugar cane [112].

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